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Overexpression of P-glycoprotein (Pgp) in breast and other cancers is thought to be largely responsible for the development of multidrug resistance to chemotherapy. Pgp actively extrudes various chemotherapeutic drugs out of cells and may also regulate Cl^- channels. The goal of our research is to investigate the relationship between Pgp structure and its multiple functions. Here, we examine the mechanism of generating Pgp C-terminal half topology. We conclude that (1) transmembrane segment (TM) 8 can insert in the membrane in an orientation opposite from what is predicted by hydropathy analysis, (2) the folding of TM7 may influence the membrane orientation of TM8, and (3) the mechanism by which positively-charged amino acids following TM8 decrease the generation of an alternate topology is not related to the net charge of these amino acids. In addition, we describe a novel approach to examine the relationship between Pgp substrate binding and Pgp topology.

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PI - Signature Date

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INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Chemotherapy has been widely used to treat cancers. However, chemotherapy often fails because breast and other cancers become resistant or are intrinsically resistant to various unrelated chemotherapeutic agents. This has been described as multidrug resistance or MDR (1,2). The overexpression of a 180-kDa protein, P-glycoprotein (Pgp), is thought to be largely involved in causing MDR. However, our understanding of how Pgp confers MDR is incomplete. The focus of this research is to examine the relationship between Pgp structure and function to gain insight into the mechanisms involved in chemotherapy failure for the treatment of breast cancer and other malignancies.

I. Structure of P-glycoprotein

P-Glycoprotein is a member of a large superfamily of transporters known as the ABC (or ATP-binding cassette) superfamily which includes other important proteins such as the cystic fibrosis transmembrane conductance regulator (1,2). This superfamily is generally characterized by a structural motif which contains 2 homologous halves, each consisting of six putative transmembrane segments followed by a nucleotide-binding domain. Although a recent structural study determined Pgp structure at 2.5nm resolution by electron microscopy (3), the detailed structure of Pgp remains elusive due to technical limitations. However, several other approaches have been attempted to understand the topological folding for Pgp. From those studies, multiple topologies have been observed for Pgp (4-9). However, agreement over one or more topologies has been lacking (10-12).

In our previous reports (see Annual Report for 1994-1995 and 1995-1996), we demonstrated using a cell-free system that C-terminal half (C-half) Pgp has at least two topological orientations in microsomal membranes derived from endoplasmic reticulum (ER). In addition, the regulation of generating multiple topologies was investigated. We showed that the generation of Pgp topologies was in part determined by the charge distribution flanking putative transmembrane segment (TM) 8. In this report, we further investigated the detailed topology of C-half Pgp and the mechanism by which multiple Pgp topologies are generated. Based on these and previous results, it may be possible to "lock" Pgp into one topology and examine how Pgp functions are affected.

II. Function of P-glycoprotein

Numerous studies have shown that Pgp actively transports a diverse group of substrates out of cells (for a review see refs. 1 and 2). However, the detailed mechanism by which Pgp transports these substrates remains unresolved. In addition to its transport function, Pgp has been reported to express a second function. Evidence is now accumulating in support of Pgp functioning as a Cl^- channel regulator (13-15). Initially,

Pgp expression was found to be associated with swelling-activated Cl^- currents ($I_{Cl,swell}$) (16-17). However in an attempt to confirm these initial findings, several groups found that $I_{Cl,swell}$ was independent of Pgp expression (13, 18-20). Recently, two experimental findings strongly support a relationship between Pgp and $I_{Cl,swell}$. First, $I_{Cl,swell}$ was shown to be regulated by PKC phosphorylation of Pgp (14). Second, we and others have demonstrated the $I_{Cl,swell}$ can be inhibited by the anti-Pgp monoclonal antibody C219 in Pgp-expressing cells (13,15,21; see also Annual Report 1994-1995). In addition, Vanoye *et al.* (15) showed through detailed electrophysiological experiments that Pgp is not a swelling-activated Cl^- channel, but likely a Cl^- channel regulator.

Previously, we reported experiments which allowed us to gain insight into the channel(s) underlying $I_{Cl,swell}$ (see Annual Report, 1995-1996). It has been suggested that $I_{Cl,swell}$ could be accounted for by the expression of a single type of Cl^- channel (22). Contrary to this hypothesis, Vanoye *et al.* (15) have recently reported that different Cl^- channels in different cells constitute $I_{Cl,swell}$. Thus, it remains unclear whether $I_{Cl,swell}$ can be accounted for by a single channel type or whether different Cl^- channels exist in the plasma membrane. In order to facilitate our interpretation of future studies involving the evaluation of Pgp function with Pgp "locked" into one topology, we investigated the biophysical characteristics of $I_{Cl,swell}$ in isolated rat hepatocytes. We found that (1) outwardly-rectifying Cl^- selective currents were activated upon cell swelling, (2) current activation did not require intracellular ATP, (3) the currents displayed little inactivation at highly positive voltages, (4) DIDS and NPPB blocked $I_{Cl,swell}$, and (5) anion currents displayed the selectivity sequence $SCN^- > I^- \cong NO_3^- \cong Br^- > Cl^-$ > Gluconate. Further experiments were planned to characterized $I_{Cl,swell}$ in isolated rat blood cells and compare them to our previous results with rat hepatocytes. However, these experiments have been deferred in order to focus on the topological analysis of Pgp and to begin the initial characterization of Pgp function in cells expressing "locked" conformations of Pgp.

BODY OF ANNUAL REPORT

METHODS

I. cDNA constructs and site-directed mutagenesis

For studies related to understanding the mechanism of Pgp topogenesis, we created truncated Pgp molecules which contain the TM segment and its flanking regions. A reporter containing a putative N-linked glycosylation consensus sequence was linked to the C-terminal end of most of the Pgp molecules (except for TM8-12; see below). These chimeric proteins were named by the truncated Pgp molecule followed by the reporter (e.g., TM7-8R represents TM7 and TM8 with its flanking regions followed by the reporter). The following constructs were created: TM7-8R, TM8R, TM8-12, and TM1/8R.

We used the recombinant PCR method (23) for the chimeric proteins TM1/8R, TM8R, and TM8-12. Essentially, PCR was performed with a chimeric primer and a second primer to amplify a particular sequence of interest. Then, the PCR product was purified from an agarose gel and utilized as a primer for a second PCR reaction. Since the chimeric primer contains a sequence complementary to a region that is not contiguous with the amplified sequence from the first PCR, one is able to combine different sequences together. Table 1 lists the primers and templates used to generate chimeric sequences.

TABLE 1

CONSTRUCT	CHIMERIC PRIMER (5'→3')	SECOND PRIMER (5'→3')	TEMPLATE
TM1/8R	(PCR1) GAATTCAACCA-TGTAT	(PCR1) GCTTCAGACAT-CTTTAATACT (PCR2) SP6†	(PCR1) pGHa-PGP-C6 (PCR2) N3R*
TM8R	(PCR1) TTCATCATCAG-TATTCTCCATCA-CG	(PCR1) SP6† (PCR2) T7†	TM1/8R
TM8-12	(PCR1)TTCATCATCAG-TATTCTCCATCA-CG	(PCR1) SP6† (PCR2) T7†	pGHaPGP-C6

* construction of N3R was described by Zhang *et al.* (6).

† SP6 and T7 primers are universal primers

After synthesis and isolation, the final PCR products were cloned using the pGEM-T-easy vector. For TM1/8R and TM8R, a cassette containing the TM regions was linked to a reporter and subcloned into pGEM-4z vector. For TM8-12, the PCR product was subcloned directly into pGEM-4z vector.

To create TM7-8R, an insert containing the reporter from TM1/8R (*Gsu*I/*Hind* III fragment) and an insert containing TM7 and TM8 from pGHaPGP-C6 (*Sac*I/*Gsu*I fragment) were ligated to pGEM-4z digested with *Sac*I and *Hind*III.

For studies involving the introduction of a point mutation, we performed site-directed mutagenesis using the recombination PCR (24) method. Briefly, PCR was performed using a primer containing the desired mutation sequence and a second primer to amply a particular sequence of interest. The PCR products were purified from agarose gel and then used as a primer for a second PCR reaction in order to create a cassette sequence containing the mutation of interest. The PCR products were then either directly cloned using pGEM-T-easy vector or first treated with T4 polymerase and then cloned into a pGEM-4z digested with *Hinc*II (for blunt end ligation). DNAs containing the desired mutation were screened by restriction enzyme digestion analysis since the mutation also introduced a novel restriction enzyme site. Table 2 shows the primer containing the point mutation and the second primers.

TABLE 2

CONSTRUCT (†)	PRIMER WITH MUTATION (5'→3')	SECOND PRIMER (5'→3')	TEMPLATE
E779Q	(PCR1) GGCAAAGCCGG-CCAGATCCTC	(PCR1) TACATCATGGC-CTGGGTG (PCR2) CAGAAAGCTT	pGHaPGP-C6
E779D	(PCR1) GGCAAAGCCGG-CGATATCCTC	(PCR1) TACATCATGGC-CTGGGTG (PCR2) CAGAAAGCTT	pGHaPGP-C6
K776E	(PCR1) TTGGCGAAGCC-GGCGAGATC	(PCR1) TACATCATGGC-CTGGGTG (PCR2) CAGAAAGCTT	pGHaPGP-C6
S795C	(PCR1) TTCCGATGCAT-GCTC	(PCR1) TCTATTGCTTCA-GTA (PCR2) CAGAAAGCTT	cys-less pGHu-PGP-C6
S831C	(PCR1) ATAGGCTGCAG-GCTT	(PCR1) TCTATTGCTTCA-GTA (PCR2) CAGAAAGCTT	cys-less pGHu-PGP-C6

† Mutant Pgp constructs are noted as follows: e.g., E779Q represents the replacement of glutamic acid residue 779 with glutamine.

A cassette containing the mutation was then subcloned into the wild-type C-half Pgp sequence. All DNA constructs were sequenced to confirm the presence of the desired mutation and to ensure proper linkages during subcloning procedures.

II. *In vitro* transcription and translation using a cell-free system

Wild-type and mutant cDNA templates were linearized with a restriction enzyme and transcribed with either SP6 or T7 RNA polymerase in the presence of a cap analog

$m^7G(5')ppp(5')G$. Following transcription, the DNA template was removed by RQ1 DNase and RNA transcripts were purified by standard methods.

Translation of RNA transcripts was performed using the rabbit reticulocyte lysate (RRL) translation system in the presence of dog pancreatic microsomal membranes (RM) as suggested by the supplier (Promega). Translation products were centrifuged at 4°C and pellet fractions were isolated and resuspended in STBS solution (in mM: 250 sucrose, 10 Tris-HCl, pH 7.5, 150 NaCl) for further processing. For experiments requiring protease digestion and endoglycosidase treatment of membrane fractions, samples were exposed to 0.1-0.2 mg/ml proteinase K for 20 min at room temperature (unless otherwise stated in the text). After addition of PMSF (10 mM final concentration) to stop the reaction, the translation material was pelleted and washed with STBS solution containing PMSF. The pellet was resuspended in a reaction mix containing the following: (in mM) 50 sodium phosphate buffer (pH 7.6), 1.25% NP-40, 0.5% 2-mercaptoethanol, 2 PMSF, 1 unit PNGase F (or equivalent volume of water for control samples), and 0.2% SDS. After incubation for at least 1 hr, electrophoresis buffer was added and the sample was analyzed by SDS-PAGE and fluorography.

III. Construction of Pgp cDNA into pLK444M expression vector

Charged amino acid mutations were introduced into full-length Pgp by subcloning a *Stu*I/*Stu*I cassette which contains the mutation sequence into a modified full-length hamster *pgp1* Pgp. The full length Pgp was modified by the insertion of a *Sal*I linker 5' to the Pgp sequence. To subclone Pgp full-length sequence into the expression vector, pLK444M, an insert containing full-length Pgp sequence was isolated after digestion with *Sal*I and ligated to a pLK444M expression vector digested with *Sal*I. Correct orientation of the Pgp insert in the expression vector was confirmed by restriction enzyme mapping.

RESULTS/DISCUSSSION

I. Topological analysis of C-terminal half Pgp

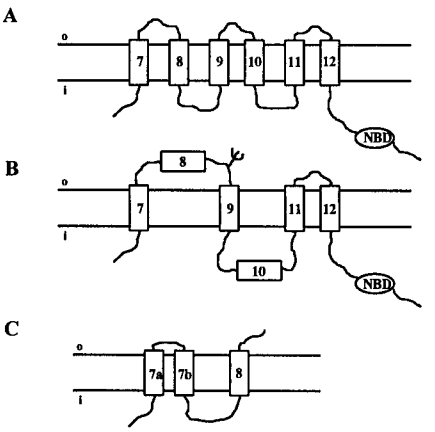
Although we proposed in the Statement of Work (see original Research Proposal) that Pgp topology would be studied in human breast cancer cells, we have decided to utilize an *in vitro* system for the topological analysis of Pgp. This cell-free system allows one to rapidly and conveniently assess the membrane topology of a protein of interest. In addition, by using molecular biological techniques, the detailed mechanism of generating a topological orientation can be easily dissected. This system has facilitated the analysis of Pgp topology as well as our understanding of the mechanism by which Pgp topology is generated. However, when Pgp mutants which potentially "lock" Pgp into a particular topology are expressed in cells, site-specific antibodies will be needed to probe the *in vivo* topology as described in the original Research Proposal. Recently, several site-specific antibodies which can aid in the determination of Pgp topology have been developed (9) and can be readily obtained. Thus, for now the generation of site-specific antibodies has been deferred.

Previously, we reported an interesting observation in which the addition of positively-charged amino acids after TM8 decreased the generation of the alternate Pgp topology (Annual Report, 1995-1996). This result suggested the possibility that Pgp could be "locked" into a particular topology. Here, we investigate further the mechanism of generating multiple Pgp topologies which will aid in the generation of specific topological populations of Pgp.

I.1. *Role of TM8 and its flanking regions in the generation of Pgp topology*

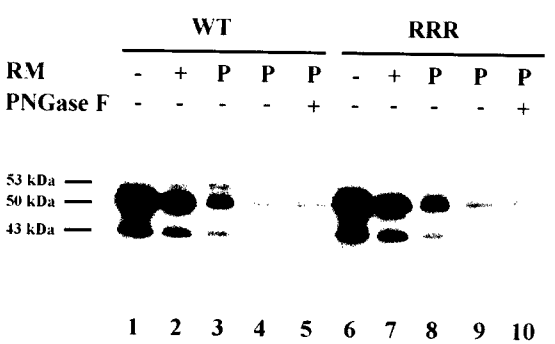
Based on models and experimental data reported from several groups (4,5,8), TM8 and its flanking regions may play a critical role in determining the Pgp topology. TM8 has been predicted from hydropathy analysis of Pgp amino acid sequence and shown by experimental results to align in the membrane with its N-terminal end facing the extracytoplasmic region (Fig.1A; refs.11-12). However, other models suggest that TM8 itself may be exposed to extracytoplasmic space (Fig. 1B; refs. 4-5) or span the membrane in an orientation opposite to the predicted topology of Pgp (Fig. 1C; ref. 8).

Figure 1. Models of P-glycoprotein C-half topology. (A) Model was derived from hydropathy analysis of amino acid sequence (2). No glycosylation is predicted. (B) Model was based on experimental results described previously (Annual Report, 1994-1995). Note the use of a N-linked glycosylation site between TM8 and TM9 (represented as a branched symbol) (C) Model based on experimental results from Bibi and Beja (8). Topology was only mapped for TM7 and TM8. TM7 was found to span the membrane twice. Putative TM segments are shown as rectangles and the nucleotide-binding domain (NBD) is represented by an oval. o = outside (or extracytoplasmic). i = inside (or cytoplasmic)



We examined the role of TM8 and its flanking region in generating multiple topologies by first determining the membrane orientation of TM8. A RNA transcript encoding TM7 and TM8 followed by a glycosylation reporter sequence was created and designated TM7-8R (see Methods, section I.). The reporter contains a consensus sequence for N-linked glycosylation and has been shown to lack topogenic signals (6). If the reporter is glycosylated, one can conclude that the reporter sequence is exposed to the ER lumen. After translation using a cell-free system, TM7-8R was found to be associated with microsomal membranes and glycosylated (Fig. 2).

Figure 2. Membrane insertion and glycosylation of TM7-8R and TM7-8(RRR)R. Translation was performed in the absence or presence of microsomal membranes (RM). Then, the reaction mix was centrifuged to isolate the membrane-associated proteins (pellet fraction or P). The membrane-associated fraction was then treated with the endoglycosidase, PNGase F (see Methods). Translation of RNA encoding TM7-8R is shown on lanes 1-5, while results using TM7-8(RRR)R RNA are shown on lanes 6-10. Note the presence of a 53-kDa glycosylated band for TM7-8R (lanes 2-4), which is absent for TM7-8(RRR)R (lanes 7-9).



To further examine TM7-8R topology, microsomal membranes were treated with proteinase K and the remaining products were analyzed on SDS-PAGE. Figure 3 shows a 40-kDa glycosylated band as confirmed by endoglycosidase F treatment (lane 2). This size corresponds in molecular weight to a glycosylated peptide consisting of TM8 and the reporter.

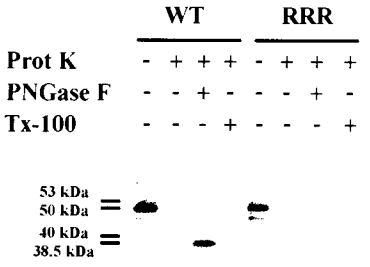


Figure 3. Membrane topology of TM7-8R and TM7-8(RRR)R. Membranes were isolated and treated with proteinase K in the presence or absence of triton X-100 (Tx-100). Some protease-resistant fragments were further treated with PNGase F (see Methods).

Taken together, these results suggest that TM8 is inserted in the membrane with its N-terminal end facing the cytoplasmic space. Interestingly when three arginines were added after TM8 (named TM7-8(RRR)R) as was done previously for the C-half molecule (see Annual Report, 1995-1996), there was little or no glycosylated band present (Fig. 2, lane 7-9). The absence of a glycosylated band was not a result of inefficient membrane targeting since the translated products were membrane-associated (Fig. 2, lane 8). When membranes containing TM7-8(RRR)R were treated with proteinase K, virtually no 40-kDa product was observed (Fig. 3, lane 6). The results with TM7-8(RRR)R suggest that the addition of positive charges after TM8 prevents both the N-terminal end of TM8 and the reporter from reaching the cytoplasmic and extracytoplasmic spaces, respectively.

Based on the results above, the biogenesis of TM7 may contribute to the membrane orientation of TM8. To accommodate the unpredicted orientation of TM8, TM7 would have to fold twice in the membrane (as hypothesized by Bibi and Beja, ref. 8). Alternatively, TM8 may act as a signal sequence and insert into the membrane while TM7 remains in the cytoplasmic space. To test whether TM7 determines the orientation of TM8, we created a chimeric peptide that consists of TM1 and TM8 followed by a reporter molecule (designated TM1/8R). A total of 4 N-linked glycosylation consensus sites are present with 3 located in the loop following TM1 and 1 present in the reporter sequence. After translations, we observed 3 N-linked oligosacchrides attached to TM1/8R as determined by limited endoglycosidase F treatment (Fig. 4).

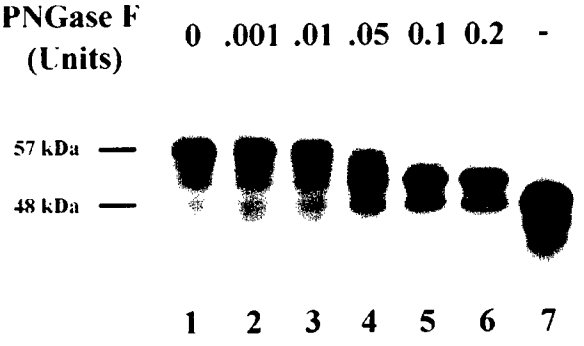


Figure 4. Limited PNGase F treatment of TM1/8R. Membranes were isolated and treated with PNGase F at the concentrations listed in the figure. Lane 7 represents TM1/8R translated without RM and served as a control for unglycosylated TM1/8R.

It was noted that PNGase F failed to remove all sugars (Fig.4, lane 6). However, all sugars were removed by treatment with endoglycosidase H (data not shown). After membrane treatment with proteinase K, a 20-kDa glycosylated band consistent in size with a protected fragment containing amino acids from TM1 to TM8 was observed (Fig. 5, lane 1). However, a minor glycosylated protease-resistant fragment of 39 kDa was also present. This minor band likely represents TM8 and the reporter since the mobility on SDS-PAGE was similar to the protease-resistant fragment from TM7-8R (compare with Fig. 3, lane 2). To confirm this hypothesis, a comparison between the protease-resistant fragments from TM7-8R and TM1/8R and a peptide consisting of TM8 and the reporter will be performed and is in progress. In summary, the results suggest that TM8 can insert in the membrane in an orientation opposite to what was predicted by hydropathy analysis. The folding of TM7 likely determines in part the membrane orientation of TM8.

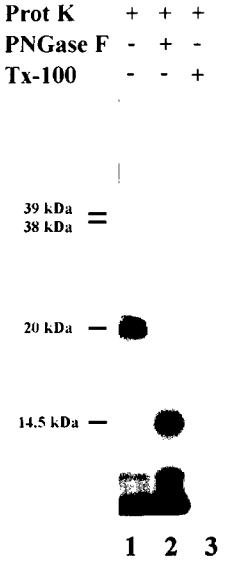


Figure 5. Proteinase K treatment of microsomal membranes containing TM1/8/R. Membrane-associated fraction of TM1/8R was treated with proteinase K in the absence (lane1) or presence of triton X-100 (lane 3). Membrane-protected fragments were further treated with PNGase F and analyzed on SDS-PAGE.

I.2 *Mechanism of decreased alternate topology by the addition of charged amino acids after TM8*

Previously, we demonstrated that the addition of positively-charged amino acids immediately after putative TM8 decreased the generation of Pgp alternate topology (i.e., glycosylated band). It was speculated that the mechanism of decreased generation of Pgp alternate topology was due to the increased ability of TM8 to insert and anchor in the ER membrane. This hypothesis was based on the notion that positively-charged amino acids tend to be commonly distributed in cytoplasmic loops, adjacent to TM regions (known as the positive-inside rule; ref. 25). Our results led to the idea that Pgp could be "locked" into a single topology (or a topology favored over another). To assess the functional consequences of expressing a Pgp molecule that favors a particular topology, we designed control charge mutations which were predicted to have no effect on Pgp topology generation. These control mutations would be necessary to determine if changes in function could be attributed to the change in topology or the mutation *per se*.

In these regards, we compared E779R mutant (glutamic acid replaced by arginine at amino acid 779) with E779D and E779Q which replaces a negatively-charged amino acid with either a similar charge or a neutral charge, respectively. To our surprise, analysis of membrane-associated peptides revealed that the replacement of glutamic acid 779 with either arginine, aspartic acid, or glutamine had similar effects of decreasing Pgp topology (Fig. 6). This suggests that the mechanism involved in decreasing Pgp topology by altering amino acid 779 is irrespective of the type of charge present. Amino acid 779 could potentially lie in a critical area required for proper folding of Pgp.

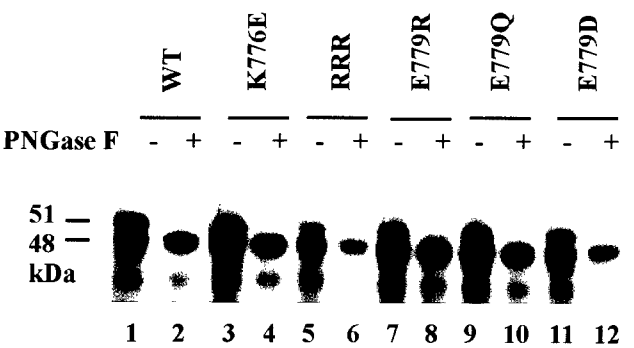


Figure 6. Effect of charged amino acids flanking TM8 on Pgp C-half topology. Membrane were isolated after translation of wild-type C-half Pgp (WT) and several charge mutants (described in the text). Membranes were treated with or without PNGase F and analyzed by SDS-PAGE. RRR mutant was previously described in Annual Report, 1995-1996. Basically, 3 arginines were inserted after TM8 following amino acid residue 776. Lanes 1 and 2: shorter exposure times were used in order to distinguish individual bands.

We also tested the effect of replacing lysine 776 with glutamic acid (K776E) which immediately follows TM8. We found virtually no effect on the proportion of glycosylated to unglycosylated bands when comparing wild-type with K776E Pgp (Fig.6, lanes 3-4). For all mutants studied, the proportion of glycosylated and unglycosylated bands may need to be examined by densitometry in order to make quantitative estimates for comparisons between the various mutants and wild-type C-half proteins. Although our

results do not clarify the precise mechanism by which amino acids flanking TM8 alters Pgp topology, they nevertheless prompt the need to determine the functional consequences of these "topological mutants" expressed in cells.

I.3 *Topology of the cysteine-less C-half Pgp*

Currently, there is controversy over the topology of Pgp. A study using site-directed antibodies and immunohistochemistry demonstrated the presence of a Pgp topology different from hydropathy-predicted model in a MDR cell line (9). However, two recent reports argue that the topology of Pgp is the same as predicted by hydropathy analysis (11-12). One study introduced an epitope tag into various regions of Pgp and determined the sidedness of the tag by immunofluorescence microscopy (11). However the sidedness of the epitope, when placed in the controversial region of the loop between TM8 and TM9, could not be determined using this method.

In contrast, Loo and Clarke (12) were able to reintroduce a cysteine by point mutation just before TM9 (amino acid 831) in a cysteine-less (cys-less) Pgp molecule and determine that its location was cytoplasmic. It is possible that the introduction of a cysteine altered the generation of Pgp topology and thus prevented exposure of this cysteine residue 831 from reaching the extracytoplasmic space. To test the hypothesis, we obtained a cys-less Pgp cDNA and created a C-half construct with a reintroduced cysteine at position 831 (S831C). In addition, we also reintroduced a cysteine at position 795 in a C-half cys-less Pgp construct (S795C). This mutation was reported to cause alterations in Pgp biogenesis (12). Creation of S795C construct is in progress. However, a DNA construct encoding S831C has been created and the results are discussed below.

Translation of RNA transcripts encoding both C-half cys-less Pgp (wild-type) and S831C resulted in glycosylated C-half products in microsomal membranes (Fig. 7, lanes 2 and 6). Our preliminary results indicate that a topology different from hydropathy-analysis could be generated for both wild-type and S831C C-half cys-less Pgps using a cell-free system.

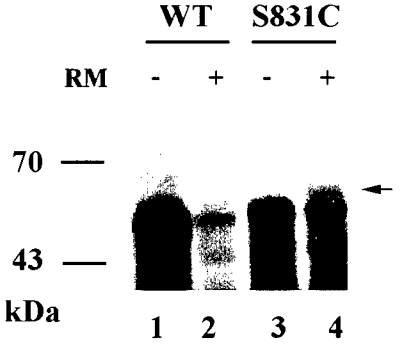


Figure 7. Topology of cys-less wild-type and S831C Pgps. A cys-less C-half Pgp (WT) and a cys-less C-half Pgp with a cysteine reintroduced at amino acid 831 (S831C) was translated in the presence or absence of microsomal membranes (RM). The reaction was then analyzed by SDS-PAGE. The arrow indicates a glycosylated band present for both WT and S831C proteins. Glycosylation was confirmed by PNGase F treatment (data not shown).

This suggests that the reintroduction of cysteine 831 does not alter the topogenesis of C-half Pgp in a cell-free system. In conclusion, it is still unclear why different topologies have been observed in different systems. The discrepancies with Pgp topology may be related to (1) the assay system used to determine membrane sidedness or (2) a unique process of biogenesis (e.g., a topological maturation process) for Pgp. Ultimately, obtaining high resolution structural information will be needed to resolve this controversy.

II. Functional analysis of Pgp

In our previous reports, we demonstrated that Pgp expression is associated with swelling-activated Cl^- currents ($I_{Cl,swell}$). We then examined the biophysical characteristics (e.g., ionic selectivity, blocker sensitivity) of $I_{Cl,swell}$ in isolated rat hepatocytes to facilitate our analysis of Pgp and $I_{Cl,swell}$. Currently, we have postponed the characterization of $I_{Cl,swell}$ in isolated rat blood cells because of technical issues related to specific blood cell isolation. However, a commercially available kit is available now to specifically isolate rat neutrophils. Currently, we are pursuing a potentially convenient and powerful method of assessing Pgp function which is described below.

II.1 *Detection of Pgp conformational change using an in vitro system*

Various studies have examined the effects of Pgp substrates on the structure of Pgp. Recently, limited trypsin digestion of Pgp in membrane vesicles resulted in a digestion profile of protease-resistant peptides that depended on the presence of certain Pgp substrates (Wang *et al.*, in press). The implications of this study are that the binding of Pgp substrates can alter the conformation of Pgp. Potentially, a conformational change could entail a change in Pgp topology. In this respect, we utilized our *in vitro* translation system as a means to detect conformational change in Pgp. The *in vitro* system offers a quick means to assess the topology of Pgp, and with the power of molecular biology Pgp sequence can easily be manipulated to aid in our understanding of the mechanism of substrate-mediated conformational change. In this regards, we examined the protease digestion profile of C-half Pgp in the presence of either vinblastine or ATP. We chose to examine the effects of Pgp substrates on the C-half molecule alone because we had already characterized its topology using this system.

First, we tested the effect of vinblastine on proteinase K digestion of C-half Pgp. Membranes containing Pgp were isolated and incubated in various concentrations of vinblastine for 20 min. Proteinase K was then added at maximal concentrations in the presence of vinblastine to allow complete digestion. Protease-resistant peptides were then analyzed by SDS-PAGE. Figure 8 shows an increase in the 17-kDa glycosylated band which was maximal at $\approx$ 50 μM vinblastine.

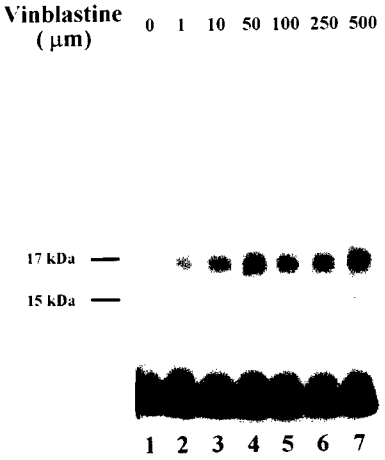


Figure 8. Effect of vinblastine on proteinase K digestion of C-half Pgp. RNA encoding pGHaPGP-C6/*Xba*I was translated in the presence of RM. Membranes were isolated and incubated in the presence of vinblastine (concentrations listed in the figure) for 30 min at room temperature. Then the membranes were treated with proteinase K for 20 min at room temperature. PMSF was added to stop the digestion and peptide fragments were analyzed by SDS-PAGE.

This effect was not apparently due to an increase in post-translational N-linked glycosylation since an increase in glycosylated product was not observed in the absence of proteinase K (data not shown). However, it is possible that vinblastine alters the activity of proteinase K. We are currently testing this hypothesis.

We next tested the effect of 50 μM vinblastine on the proteinase K digestion profile of C-half Pgp. We observed no apparent difference in digestion profile between C-half in the presence or absence of vinblastine (Fig. 9).

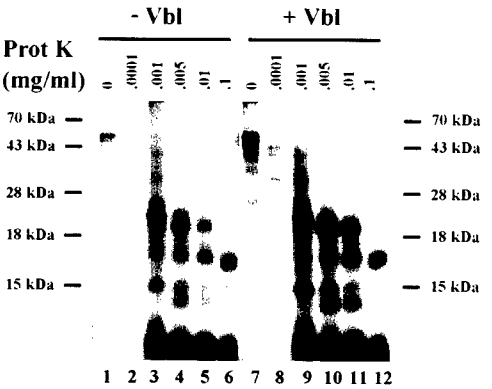


Figure 9. Effect of vinblastine on C-half protease digestion profile. Isolated membranes were treated with either 50 μM vinblastine (+ Vbl) or STBS solution (- Vbl; see Methods) for 30 min at room temperature and then treated with various concentrations of proteinase K for an additional 20 min at room temperature (in the presence of 50 μM vinblastine for lanes 7-12). The reaction was stopped by the addition of PMSF and analyzed by SDS-PAGE.

The apparent lack of effect of vinblastine on proteinase K digestion profile does not rule out the possibility that a conformational change occurred. Since our assay detects only a certain fragments of Pgp, other parts of the molecule could still be changing. Also, it is assumed that vinblastine bound to the *in vitro* synthesized C-half molecule. Both half molecules may need to be present for drug binding.

In addition to vinblastine, we tested whether Mg^{2+}-ATP affected the C-half protease digestion profile. Instead of proteinase K, we used trypsin as the protease to

digest C-half Pgp. In addition, we used a cys-less C-half construct that contained both the TM domain and nucleotide-binding domain. We found that the digestion profile of both proteinase K and trypsin were virtually similar (data not shown). When we compared the trypsin digestion profile of a C-half Pgp molecule that contained both the TM domain and nucleotide-binding domain in the presence and absence of ATP, we again observed no differences in the digestion pattern (Fig.10). However, we noted that ATP reduced the activity of trypsin (previously reported by Wang *et al.*, in press).

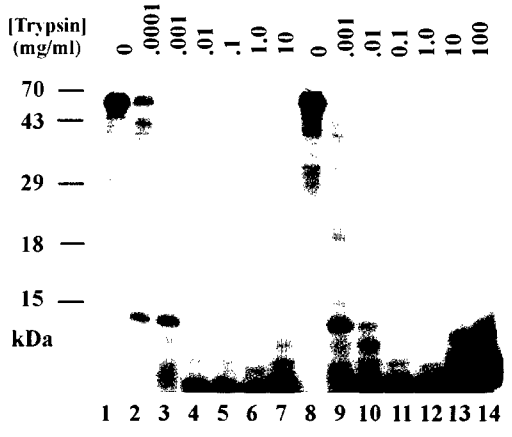


Figure 10. Effect of ATP on C-half protease digestion profile. Isolated membranes were treated with either 5 mM Tris-Cl Buffer (lanes 1-7) or 5 mM Mg^{2+}-ATP (lanes 8-14) (final concentration) for 30 min at room temperature and then treated with various concentrations of trypsin (in the presence of 5 mM Mg^{2+}-ATP). The reaction was stopped by adding PMSF and soybean trypsin inhibitor and incubating on ice for 10 min. Final products were then analyzed by SDS-PAGE.

Although our initial results suggest that conformational changes due to binding of Pgp substrates can not be detected by our system, we will attempt a few additional control studies before abandoning this project (e.g., test effect of Pgp substrates on protease enzyme activity). One potential pitfall of this project is that expression of both halves may be necessary to induce a topological change by Pgp substrates. Attempts will be made to co-express both Pgp halves and determine the effects of Pgp substrates on Pgp topology.

II.2 *Construction of Pgp topological mutants into the expression vector pLK444M*

Our results indicate that Pgp could be "locked" into a particular topology at least by introducing charged amino acids into the flanking regions of TM8. In this context, we will next determine the structural and functional consequences of cellular expression of Pgp molecules containing the mutations which were examined *in vitro*. Experiments are currently in progress to insert charge mutations (e.g., E779R, E779Q) into full-length hamster *pgp1* Pgp (see METHODS, sec. III). Then, the Pgp insert containing the charge mutation will be subcloned into the expression vector pLK444M and expressed in BALB/c-3T3 cells using the liposome-mediated transfection technique (26). We chose this approach because this transfection protocol has been successfully used in our laboratory to express other Pgp constructs without any major technical difficulties (15). Pgp structure and function will then be addressed as outlined in the original Research Proposal.

CONCLUSIONS

The results from this report indicate that (1) TM8 can insert in the membrane with its N-terminal end facing in an orientation opposite from predicted (i.e. towards the cytoplasmic space), (2) the topological folding of TM7 may influence the membrane insertion of TM8, (3) decreased generation of Pgp alternate topology by introducing a point mutation at glutamic acid residue 779 does not depend on the relative charge of the newly introduced amino acid, (4) the *in vitro* membrane topology of both cys-less wild-type and S831C mutant C-half Pgp are similar to native C-half Pgp, and (5) protease digestion profile of C-half Pgp in microsomal membranes is not apparently altered by the Pgp substrates vinblastine or ATP.

Based on our results from this report, the generation of C-half topology may be more complex than initially thought. The unexpected folding of both TM7 and TM8 may offer new insight into the topology of Pgp and the biogenesis of membrane proteins in general. In addition, our results and other recent work (27) support a model for C-half Pgp that was originally proposed by Bibi and Beja (8). The observations of multiple C-half Pgp topologies may now revolve around two topologies, the hydropathy-predicted topology and the topology proposed by Bibi and Beja (8). Since only a partial topology for the C-half molecule was established by these investigators (i.e., TM7-8; ref.8), further work is necessary to clarify the topology for the rest of the C-half molecule. Although further work will be pursued towards this end, our current main focus is to express Pgp constructs with our mutations that we created and examine the functional consequences. The construction of full-length Pgp containing charged amino acid mutations into an expression vector is currently in progress.

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APPENDIX

Progress in the M.D.-Ph.D. program

In response to the Annual Report Review for the 1995-1996 FY, I am reporting my progress in the graduate program. In November 1996 my thesis proposal was accepted by my thesis committee. Currently, I am working towards completing the research requirements for my Ph.D. thesis in the Cellular Physiology and Molecular Biophysics Program, under the direction of both Drs. Jian-Ting Zhang (thesis advisor) and Luis Reuss (chairperson for thesis committee). I have been receiving continuous guidance and support from both of my mentors, who have been very helpful and encouraging. My projected completion of the requirements for a Ph.D. degree is November 1998.

Received 2/8/00

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